DIPLOID AND ANDROGENETIC HAPLOID HYBRIDIZATION BETWEEN TWO FORMS OF RANA PIPIENS, SCHREBER[1]

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Introduction

One of the better methods for examining nucleo-cytoplasmic relationships is to combine identical nuclei with different cytoplasms. This can be achieved in a number of ways. For example, if the gametes of two different species are brought together to form reciprocal diploid hybrids, it is expected that at least in the early stages such hybrids will have identical nuclei and different cytoplasms. This arises from the fact that in most cases the maternal parent contributes practically all of the cytoplasm. Differences which may appear in the development and heredity of the reciprocals can therefore be related to differences in the egg cytoplasms of the two parent forms. If such cytoplasmic differences are observable and measurable, the possibilities are obvious.

The same end is achieved by combining the male nucleus of one species, subspecies or race with the nucleus-free egg cytoplasm of the same species and another species, subspecies or race. If the androgenetic or merogonic[2] homospermic haploid and heterospermic haploid resulting from this procedure show dissimilarities, these must be related to cytoplasmic differences.

It is scarcely necessary to point out that the results of such procedures seldom if ever satisfy the preconceived possibilities. The results from reciprocal diploid hybrids may be limited by an incompatibility of combined nuclei or nuclei and cytoplasms; or, where this is not the case, by the absence of sufficient cytoplasmic difference to produce an effect. And heterospermic haploids are usually less satisfactory. In the best of circumstances haploid organisms develop poorly and can only be produced in a limited group of materials. Apparently as a result of

[1] Data obtained, in part, from experiments performed during tenure of National Research Fellowship at Princeton University.

[2] The term androgenetic refers to the development of the *whole* egg with only the male nucleus functional; merogonic refers to the similar development of an egg-*fragment* (Wilson, 1925).

a high degree of incompatibility between nucleus and cytoplasm, the development of most androgenetic species hybrids is extremely abnormal and ceases in the earliest stages.

Despite such results, the possibility remains that hybridizations with heretofore untried material such as the North American Salientia may reveal one or more compatible combinations with the desired qualities. Experiments to test this possibility have been made and the following pages report one such investigation. Two distinct but closely related forms of the genus *Rana* have been combined reciprocally to form diploid and androgenetic haploid hybrids. The results form an interesting addition to the existing data on nucleo-cytoplasmic relationships.

The author is very grateful to Prof. G. Fankhauser for helpful suggestions and criticisms.

Materials

The gametes for these hybridization experiments were derived from two distinct forms of frog, one collected from the meadows of northern Vermont, the other from the immediate vicinity of Philadelphia. Both forms are commonly referred to as *Rana pipiens* and possibly represent different races or subspecies of that species. More attention will be given to their probable relationship in the discussion.

That the two forms are distinct is indicated by their general characteristics (Figs. 1 and 2), and also by the results of these experiments. These same features also indicate that the two are closely related. Therefore, as a temporary assumption and to facilitate the description of the experiments, the frogs are being considered as northern and southern forms or races of the same species. As such they will be referred to in the succeeding pages of this report.

A brief description, supplemented by Figs. 1 and 2, will indicate their major differences and similarities.

The northern form (from northern Vermont) is generally larger and, relative to its body size and weight, it has shorter jumping legs than the southern form. The head is obtuse; the vocal sacs on the male are less apparent; the dorso-lateral folds are broad; the skin is thick; the palmation is full. Distinctive features of pigmentation include spots that are larger and surrounded by a green or yellow border; the cross-bars on the tibia are generally complete; the posterior border of the thigh is marked by black spots on a continuous white background; the tympanum does not show a central light spot with the same clarity as in the southern form.

The southern form (from the vicinity of Philadelphia) is generally smaller and, relative to its body size and weight, its legs are longer. The head is more accumate; the vocal sacs are thin-walled and usually

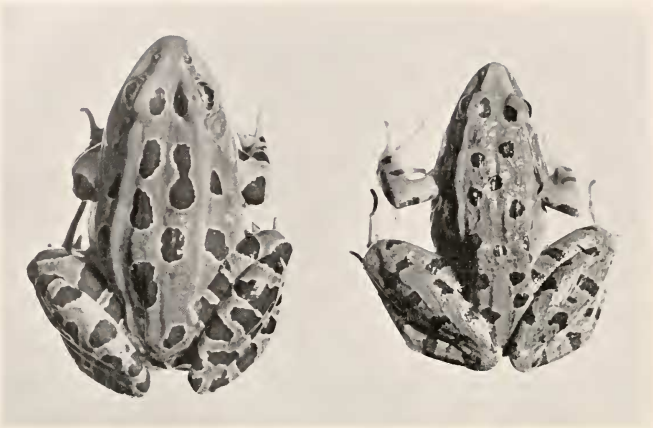

FIG. 1. Photographs of representatives of northern (left) and southern (right) forms of *R. pipiens* used in these experiments.

FIG. 2. Photographs showing pigmentation of jumping legs, northern (left) and southern (right).

apparent; the dorso-lateral folds are narrow; the skin is thin; the palmation is shallow. Distinctive features of pigmentation include spots that are smaller and, under the same laboratory conditions as the northern form, not surrounded by clear borders; the cross-bar markings on the tibia are generally interrupted along the dorso-lateral surface; the posterior surface of the thigh is marked by white spots on a continuous black background; and the tympanum generally shows a central light spot.

Methods

The eggs were obtained in every case from frogs which had been induced to ovulate by frog pituitary injections. As much care as possible was taken to avoid removing the eggs in the immature or over-ripe condition. The sperm for insemination were obtained by macerating the testes in 10 per cent Ringer's solution and every precaution was taken against contamination of one suspension with sperm from another.

The eggs of each frog were inseminated in two batches, the first with sperm of the same form, the second with sperm of the other form. Thus, in all, four batches of eggs were inseminated. An interval of 15 to 20 minutes was allowed to elapse between each insemination to provide time for removing the egg pronucleus from a number of eggs of each batch. By this procedure 8 different types of embryos were produced. These are listed below with the designation used for each in the balance of this report.

Homospermic diploids of the northern form	*n*
Homospermic haploids of the northern form	*n/2*
Heterospermic (hybrid) diploids from eggs of northern form and sperm of southern form	*ns*
Heterospermic (hybrid) haploids from cytoplasm of northern form and nucleus of southern form	(*n*)*s/2*
Homospermic diploids of the southern form	*s*
Homospermic haploids of the southern form	*s/2*
Heterospermic (hybrid) diploids from eggs of southern form and sperm of northern form	*sn*
Heterospermic (hybrid) haploids from cytoplasm of southern form and nucleus of northern form	(*s*)*n/2*

The egg pronucleus was removed with a fine glass needle as described in a previous report (Porter, 1939). Adequate numbers of pure and hybrid haploids were thus easily prepared (Table I).

All embryos were kept under identical conditions of temperature (19.4° C.) and space. In fixation of representative forms for a permanent record, a mercuric chloride, acetic acid, and formaldehyde mixture was generally used. The same sequence and time intervals were observed in fixation as had been observed in fertilization. Thus it was

assured that all animals fixed at the end of a period of time were of the same age.

Results

The description which follows is based upon observations made in the experiments listed in Table I. The possibility that the same results could occur by coincidence in all four series of crosses is slight if not negligible. The analysis is confined to such characteristics as were ap-

Table I [3]

Exp.	Date	Number of homospermic haploids produced	Number of heterospermic (hybrid) haploids produced	Treatment
1.	Jan. 9, 1939	23 *n/2* 21 *s/2*	41 (*n*)*s/2* 35 (*s*)*n/2*	Preliminary comparison of living animals made throughout development. Representative embryos fixed at end of 3, 5, 7, 9, 10 and 11 days.
2.	Jan. 17, 1939	29 *n/2* 37 *s/2*	30 (*n*)*s/2* 44 (*s*)*n/2*	Living animals compared throughout development. Representative forms fixed at end of 2, 3, 4, 5, 6, 7 and 8 days. Diploid hybrids and controls carried through metamorphosis for examination of inheritance.
3a.	Feb. 15, 1939	37 *n/2* 24 *s/2*	48 (*n*)*s/2* 43 (*s*)*n/2*	Living animals compared throughout development. Special attention given to gastrulation and neural tube formation. Representative forms fixed at end of 36, 43, 48, 51, 53, 55, 57, 59, and 61 hours and at 3 and 4 days.
3b.	Feb. 15, 1939	21 *n/2* 45 *s/2*	36 (*n*)*s/2* 41 (*s*)*n/2*	Living animals compared. Special attention given to study of older stages. Material fixed at end of 32, 54, and 60 hours and 3, 5, 6, 8, 9, and 10 days.

[3] The same females were used as a source of eggs for experiments *3a* and *3b*. Otherwise, different parents were used in each cross.

parent from external examination and only those characteristics which were uniformly shown by the animals in all four groups are stressed in the succeeding paragraphs.

For greater clarity the description of the 3-day-old embryos is presented first. With the differences of these in mind the descriptions of the younger and older stages have greater meaning.

Three-day-old Embryos

The following account is illustrated by the outline drawings in Fig. 3 and to them reference is constantly made.

The homospermic (control) diploids of the two races develop at approximately the same rate at 19.4° C. and, stage for stage, are comparable at the end of 72 hours. The differences, though real, are very slight and were clearly recognized only after repeated examination of material available. The northern diploids compared with the southern diploids show larger gill plates, a larger sense plate and larger mucous glands. Relative to body size the head of *n* is the larger. The neural tube is broader and stands up more distinctly in *n*. The tail-bud in *n* is smaller and directed more dorsally than in *s*, thus creating a deeper depression in the back of *n*. In relation to head size, the abdomen of *s* is larger than that of *n*. To these differences it can be added that the head flexure dorsal to the posterior margin of the gill plate is more pronounced in *s* than in *n*.

The homospermic (control) haploids of the two races, as is normal for haploids, are retarded in their development. Compared with each other they show in an exaggerated form the same differences that were given for the diploid controls.

The heterospermic (hybrid) diploids show approximately the same rate of development as the homospermic diploids and as each other. They differ in body proportions and show in accentuation the differences which are difficult to see between the pure diploids of the two forms. A greater proportion of *ns* consists of head structures than in the reciprocal hybrid. Conversely, a greater proportion of *sn* consists of abdomen and tail-bud. The mucous glands and sense plate are larger in *ns* and, posterior to the medulla, *ns* shows a smaller neural tube which terminates in a smaller and more dorsally directed tail-bud.[4]

The heterospermic (hybrid) haploids show in most exaggerated form the differences which have been referred to as existing between control diploids and haploids and more distinctly between the hybrid diploids. It is readily apparent that oral suckers, gill plates, and sense plate are greatly enlarged in *(n)s/2*. Relative to head size, the abdomen and tail-bud of *(s)n/2* are much larger than the same structures of *(n)s/2*. It can be further noted that the head of *(s)n/2* is flexed more ventrally than *(n)s/2* and the back of the latter is convex while in the former it

[4] If these experiments had been confined to the production and study of diploid hybrids, it is doubtful if the differences would have been considered great enough to warrant any conclusions. Supported by the evidence from androgenetic hybrids, however, the significance of the differences is unquestionable.

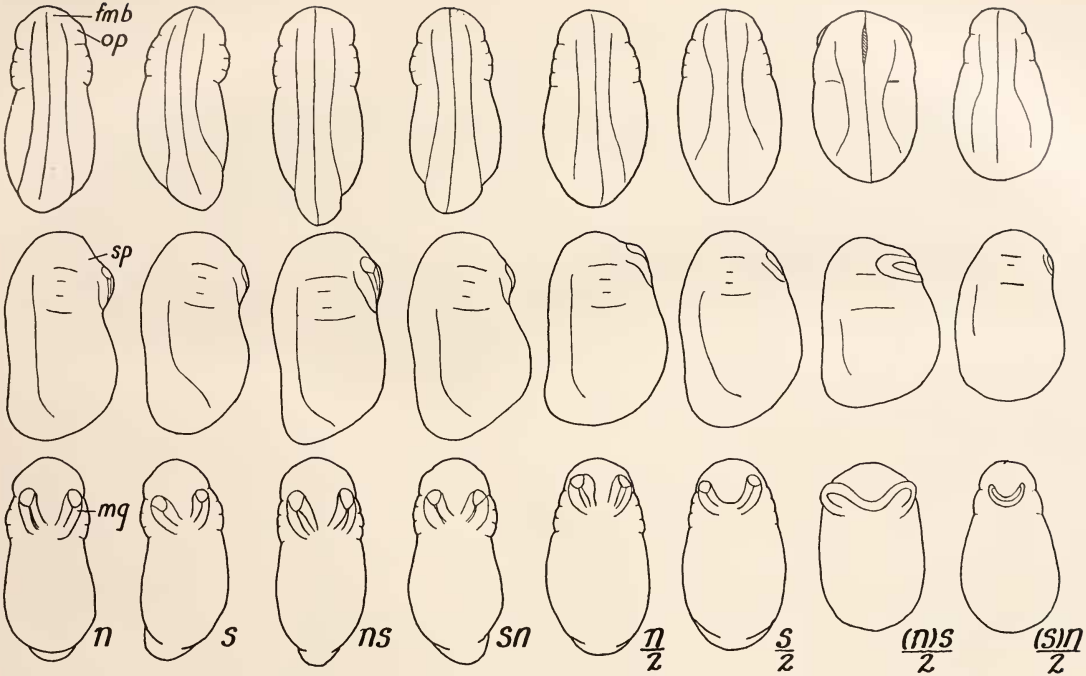

FIG. 3. Outline camera lucida drawings of dorsal, lateral and ventral views of 72-hour-old embryos. From left to right the drawings represent: *n*, homospermic diploid control of northern form; *s*, homospermic diploid control of southern form; *ns*, heterospermic diploid from northern egg and southern sperm; *sn*, heterospermic diploid from southern egg and northern sperm; *n/2*, homospermic haploid control of northern form; *s/2*, homospermic haploid control of southern form; *(n)s/2*, heterospermic haploid (androgenetic hybrid) from northern cytoplasm and southern sperm; *(s)n/2*, heterospermic haploid from southern cytoplasm and northern sperm. Magnification, 12.5 ×. The same sequence and magnification are observed in Figs. 4, 5, 6, and 7.

It can be seen that head structures (fore- and mid-brain regions, *fmb*, optic vesicle outpushings, *op*, sense plate, *sp*, and mucous gland, *mg*) are larger in *n*, *ns*, *n/2*, and *(n)s/2*, than *in s*, *sn*, *s/2*, and *(s)n/2* respectively. The tail-bud, *t*, tends to be larger in the

is concave. These differences are the expression of the decidedly dissimilar embryology of the two reciprocal heterospermic haploids and not a difference in age or stage.

Summary.—In general the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such differences, only slightly apparent in the pure diploid controls, become progressively more accentuated in the homospermic haploids, the heterospermic diploids and in the heterospermic haploids.

It is of interest to observe now the earlier and later expressions of these general differences as shown by an examination of the earlier and later stages in the ontogeny of the various combinations.

Neural Tube Formation

The description under this heading is derived from a comparative study of living material and of representative embryos of the eight different types fixed at intervals of two hours from 51 to 61 hours after insemination. Illustration is provided by outline figures 4, 5, and 6 which are respectively representative of developmental stages reached at the end of 55, 59 and 61 hours.

The homospermic diploids, during this period, are very similar both in character and rate of development. As the neural plate is outlined, it becomes apparent that its anterior portion plus the sense plate are larger in *n* than in *s*. These differences increase in clarity as the neural folds are elevated and gill plates appear (Figs. 5 and 6). At this latter stage, *s* flattens dorsally and shows a greater elongation of that portion of the neural groove posterior to the gill plates. At the same time the neural plate and folds are more distinctly elevated in *s*. Although the neural plate and folds may be outlined in *s* slightly in advance of *n,* the closure of the folds is more rapid in the latter. During neurulation the blastopore of *s* is bounded laterally by distinctly thickened lips.

The homospermic haploids show in exaggerated form the slight differences existing between the diploid controls. In equivalent stages (55 hours) the neural folds of *n/2* are thicker, the sense plate and other primordial head structures are larger than in *s/2,* whereas the latter shows a greater elongation of the neural plate, especially that portion of it determined to be spinal cord. In *s/2* the neural folds show a greater elevation, the dorsal surface straightens or flattens out, and pronounced lateral lips bound the blastopore. Besides stage-for-stage structural differences, the differences in rates of separate morphogenetic processes are also accentuated. For example, it is noted that *s/2* completes gas-

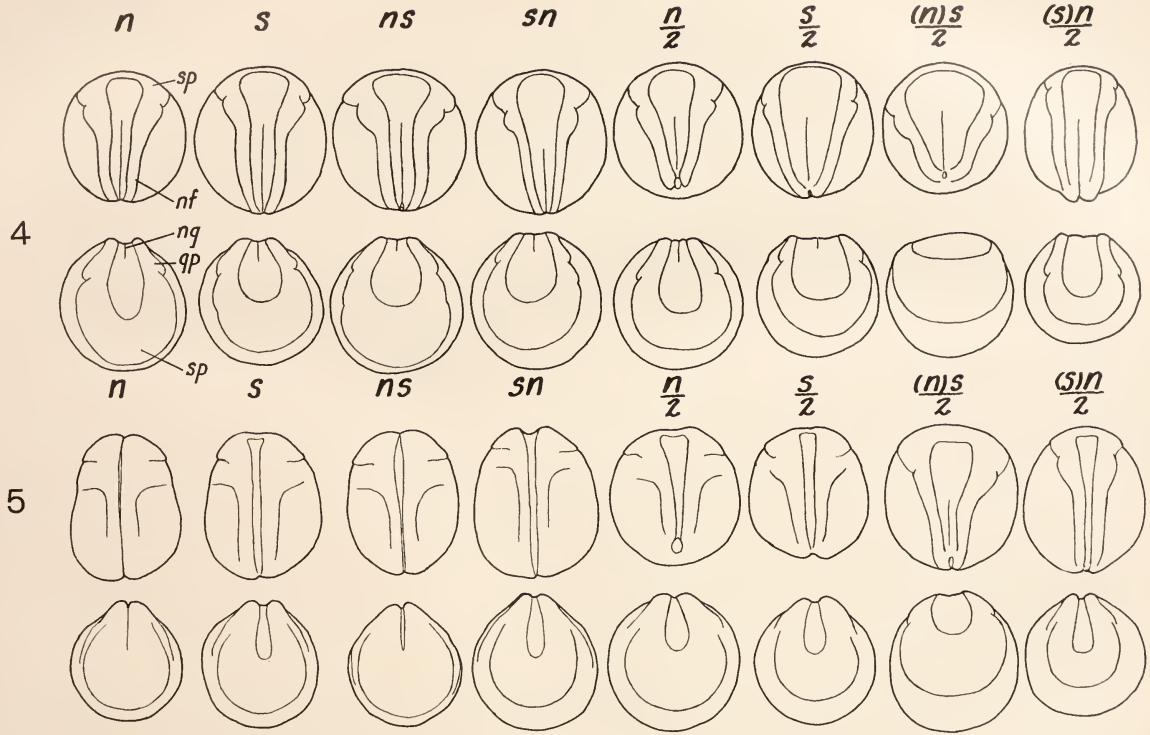

Fig. 4. Dorsal and anterior views of 55-hour-old embryos. It can be noted that, relative to size of abdomen, the head primordia (sense plate, *sp.*, gill plate, *gp.*) of *n*, *ns*, *n/2*, and *(n)s/2* are larger than the same of *s*, *sn*, *s/2* and *(s)n/2*. Neural folds, *nf.* Neural groove, *ng.*

Fig. 5. Dorsal and anterior views of 59-hour-old embryos.

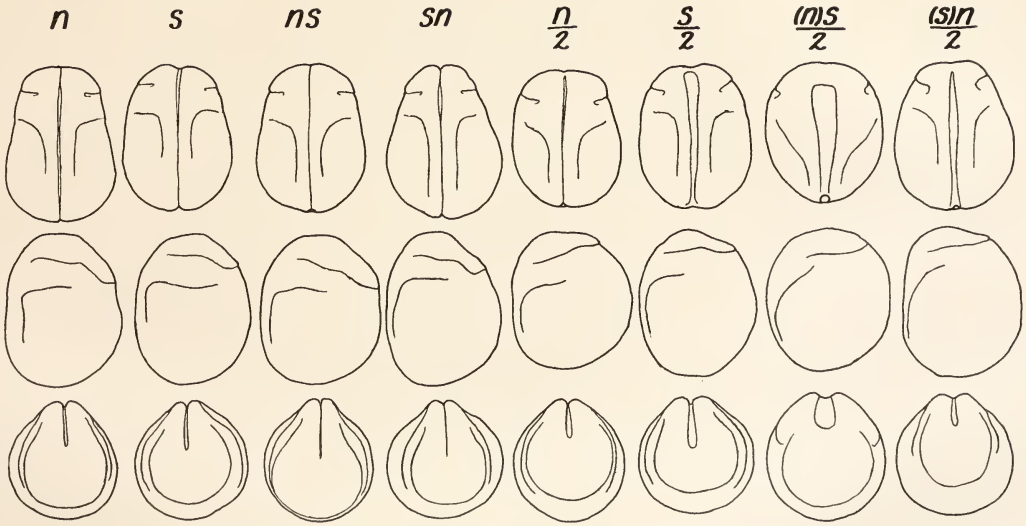

Fig. 6. Dorsal and anterior views of 61-hour-old embryos.

trulation considerably ahead of *n/2* and only slightly after *s,* whereas in *n/2* the neural folds appear to close slightly before they do in *s/2.*

The heterospermic diploids show clear-cut differences. Compared with each other in the early stages of neurulation, it is apparent that the *ns* forms have a shorter neural plate which is abnormally broad at the anterior end. The reciprocal *sn,* on the other hand, has a long and narrow neural plate. As the two differ from each other so do they differ from their maternal control diploids though to a lesser degree. Other features of dissimilarity include neural folds which are larger in *ns* than in *sn* and which are more distinctly elevated in *ns* than in the control diploid, *n.* In this latter respect they approximate the condition noted above as apparent in the paternal diploid control. As neural tube formation continues, the greater size of the head primordia and the shorter neural plate and groove are maintained in *ns.* Though the neural plate and folds are outlined almost simultaneously in these reciprocal hybrids, the folds come together in *ns* slightly before they do in *sn.* The lateral borders of the blastopore are swollen in *sn* to form lips as in *s* and *s/2.*

The heterospermic haploids show very striking differences during the development of the neural tube. At the end of 51 hours (not illustrated) (*s*)*n/2* has a dorsal flattened surface, abnormally straight from anterior to posterior ends. The neural plate is clearly outlined and is very narrow. Gastrulation has been completed, and there are extremely pronounced lips on both sides of the blastopore. Contrasting with this, the reciprocal (*n*)*s/2* is considerably retarded. The yolk plug is still apparent and the limits of the neural plate are not visible. The (*n*)*s/2* embryos are flattened dorso-ventrally and present a large, swollen appearance. By the end of 55 hours the neural plate of (*n*)*s/2* has been outlined. It is as broad as it is long and that portion designated to become neural tube is extremely short. The yolk plug persists. At this same time in (*s*)*n/2* the neural plate has lengthened and the neural folds have approximated to some extent. At 59 hours (*n*)*s/2* continues to show a short, broad neural plate, bounded by prominently elevated neural folds. This latter feature is a characteristic of *s* embryos and its appearance in these (*n*)*s/2* embryos represents the appearance of a specific paternal character. In the reciprocal it is not shown. It is a feature which will be easier of description and analysis when sectioned material is available. By 61 hours the neural folds of (*n*)*s/2* have started to approach and subsequent observations have shown that once started this process proceeds more rapidly here than in (*s*)*n/2.* At this time and later there is little elongation of the neural plate in (*n*)*s/2* and the yolk plug still persists in some cases. The sense plate and gill plates are abnormally large. The structure of the reciprocal hybrid (*s*)*n/2* at 61

hours is characterized by neural folds about ready to close, an elongate neural tube, and extremely small head primordia which foreshadow the diminutive head size of later stages.

Summary.—In summarizing, a few generalizations can be made. Those combinations which include cytoplasm of the northern race, including the diploid controls, are characterized by: (*a*) neural plates which when outlined tend to be shorter, and broader anteriorly, and (*b*) head primordia which are larger. The reciprocal combinations with cytoplasm of the southern race are, on the other hand, characterized by: (*a*) longer and narrower neural plates, (*b*) smaller head primordia, and (*c*) pronounced lateral lips on the blastopore. These differences become increasingly apparent as one compares respectively the diploid controls, the haploid controls, the reciprocal hybrid diploids, and the reciprocal androgenetic hybrids.

At one stage in the development of the neural folds it is apparent that they are more sharply delimited and distinctly elevated in the diploid of the southern form. This characteristic is repeated in the hybrid diploids and in the androgenetic hybrids containing the southern nucleus. It seems to represent, therefore, an inheritable embryonic characteristic capable of expressing itself in the foreign cytoplasm of the northern race. More careful analysis of this phenomenon is needed.

There are also to be noted slight differences in the times of occurrence and rates of the same morphogenetic processes. Relative to blastopore closure the neural plate is outlined earlier in those combinations with northern cytoplasm. Relative to time after fertilization, however, this may be later. Once clearly outlined the neural folds of the combinations with the northern cytoplasm seem to close more rapidly.

Gastrulation

This phase of the embryology of these various combinations was studied from living material and from representative forms fixed at the end of 36, 43, and 48 hours. A few differences between the gastrulae of those forms with northern cytoplasm and those with southern cytoplasm occur consistently (excepting the diploid controls where they are not sufficiently pronounced to be clearly evident) and become progressively more pronounced in haploid controls, heterospermic diploids, and heterospermic haploids. Those combinations with the cytoplasm of the southern race show a larger gastrular angle, a smaller completed blastopore, epiboly largely from the dorsal and lateral borders of the blastopore, and toward the end of gastrulation, an increasing thickening of the lateral blastopore lips. Those combinations with northern cytoplasm show a smaller gastrular angle, a larger blastopore, epiboly from all sides of

the blastopore, and thin blastopore lips. Gastrulation appears to begin earlier in *s, s/2, (n)s/2,* and simultaneously in *sn* and *ns.* Observations recorded on this feature and on the rate of gastrulation are not sufficiently extensive to be conclusive.

It would seem that the greater gastrular angle and the greater epiboly of the dorsal lip in those haploid and hybrid embryos with the southern cytoplasm are the early abnormalities related to the longer neural plate of later stages. It also appears that the thickened lateral blastopore lips of these same forms are the early expression of the larger tail-bud and somites of later stages (Bijtel, 1931). The opposites of these same features in those forms with the northern cytoplasm are probably related to the shorter neural plate and smaller tail-buds of their later stages.

Older Stages (4–10 Days)

The studies reported in this paper have been largely devoted to the younger stages hence only the most general features of the older stages will be described under this heading. Reference should be made to Fig. 7.

The homospermic diploids of 4 and 5 days continue to show the slight differences which existed between the 3-day-old embryos. In the older stages, however, these differences become increasingly subtle. Relative to body proportions, the head of *n* remains larger while the tail of *s* is more elongate and larger in relation to the rest of the embryo. The dorsal concavity of *n* persists in greater prominence than in *s.*

The homospermic haploids differ in the older stages, as they had in the earlier stages, in relative size of body parts. The differences are similar to but more distinct than those occurring between the diploid controls.

The heterospermic diploids demonstrate more clearly the perpetuation of early differences. The combination, *ns,* persists in showing at various ages a larger head with larger mucous glands and a smaller dorsally directed tail. The converse of these features are shown by the reciprocal. Such differences are retained into the later stages of development, especially the relative head and tail size. Clear-cut appearance of paternal characteristics is recognized first in stages showing chromatophore patterns.

The heterospermic haploids, as in the younger stages, show the most striking differences. It is recognized, however, that these differences are less pronounced in the older stages suggesting some regulation. It is easily noted that the head of *(n)s/2* and its component structures remains larger and the tail remains smaller and directed dorsally. The androgenetic hybrid, *(s)n/2,* on the other hand, is characterized by a

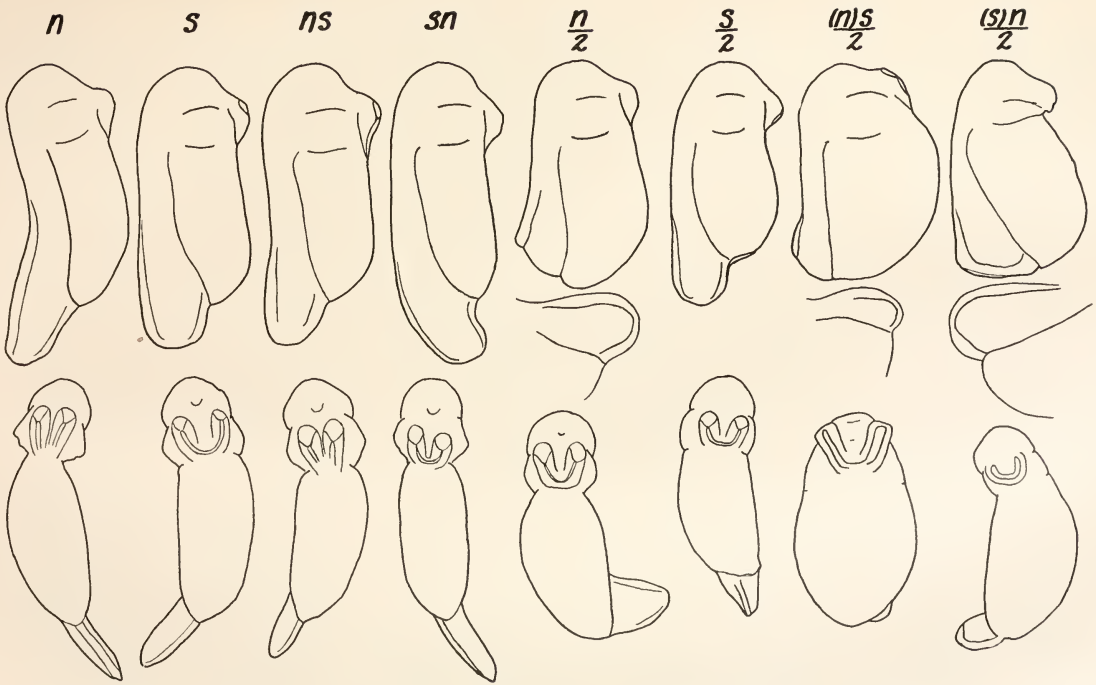

FIG. 7. Lateral and ventral views of 4-day-old embryos. It can be noted that early differences in relative body proportions persist in the various combinations.

small head and a ventrally directed tail which is referred to as larger because of the broad heavy somite mass at its base. These features were foreshadowed in the early embryonic development.

Summary.—Those combinations with cytoplasm of the southern race tend to have smaller head structures and, relative to body size, larger tails than the reciprocals with northern cytoplasm. Such features are doubtless the expression of earlier embryonic differences in the size of head primordia and tail-buds.

Survival of Various Combinations

Since representative embryos were sacrificed for fixation at various intervals, no definite data can be given to demonstrate survival value. Nevertheless, the observations made permit the following statements.

The heterospermic hybrids, in the majority of cases, develop up to and through metamorphosis. Beyond that stage no data are available.

The homospermic haploids of the two races demonstrate approximately the same viability. They continue their development, on the average, for from 8 to 12 days up to approximately stage 24 (Shumway, 1940).

The heterospermic haploids develop through the early stages showing only a small percentage of deaths. About one-fourth to one-third fail to hatch and of those which hatch the majority live for from 7 to 11 days or up to stages 22 and 23 (Shumway, 1940). They are slightly less viable than the haploid controls. In a small percentage of cases a gill circulation is established and in scattered cases growth continues sufficiently long to show the first guanophores. No differences in viability were recorded as existing between the two reciprocal combinations.

Identification of Haploids

The haploids were identified as such solely on the basis of the type of development. In an earlier study (Porter, 1939) it was shown that embryos which arise from operated eggs can be expected to develop as haploids in 90 per cent of the cases. Furthermore, such haploids were found to show certain definite characteristics when compared with their diploid controls. Hence, in these experiments, it has not been considered necessary to make a complete cytological examination of every embryo which developed from an operated egg, especially since group characteristics rather than individual characteristics have been considered. The isolated cases of diploidy which did appear among the embryos from operated eggs were readily identified by their development, cell size, etc.

In order to establish the chromosome count of the southern form,

the tail-tips of several haploids were examined. The examinations made indicated 13 to be the haploid count. This is the same as for the northern form determined in an earlier study.

Other Observations

In the case of two of the above-described experiments, surplus diploid embryos both pure and hybrid were kept for examination as older tadpoles and as metamorphosed frogs. The pure diploids showed differences characteristic of the northern and southern forms; the hybrid diploids showed blended inheritance with indications of stronger paternal influence in certain features of pigmentation. Thus despite the blending the reciprocal hybrids were distinguishable. These observations indicate that at least the differences in pigmentation between the two parent forms are related to differences in nuclear factors.

Two further observations, which, because of the small amount of evidence supporting them, must be considered as very preliminary, are briefly described. They are presented because of their interest as possible leads for experiments aimed at determining the nature of the factors responsible for the peculiar development of the hybrid embryos described above.

Since the frogs used were obtained from widely separated northern and southern points, it was considered of interest to examine the effect of high temperatures. Embryos representative of the 8 different combinations treated above were placed in a warming oven at 28°–29° C. Those combinations with cytoplasm of the southern form were not apparently damaged and developed in the typical manner, whereas those combinations with northern cytoplasm were markedly affected and only a very small percentage of the original number developed through 6 days. Controls kept at 19.4° C. developed normally.

Cytological examination of a few pure diploid 3-day-old embryos revealed some interesting differences in nuclear size and size of yolk granules. Measurements of nuclei of identical tissues of the two forms showed those of the northern to be the smaller. Measurements of the yolk granules revealed those of the northern to be much the larger.

Discussion

The discussion which follows will be confined to a consideration of the probable relationship of the animals used and to the more general aspects of the cytoplasmic and nuclear influences demonstrated. A detailed and inclusive treatment must await the accumulation of data from a more thorough study of these and similar hybrids. In a sense, then, this constitutes a preliminary report.

When the experiments were first undertaken the parents were thought to represent two distinct species. This conclusion was based on differences which the animals showed and also on the authority of amphibian taxonomists (Kauffeld, 1937; Stejneger and Barbour, 1939). An examination of the literature soon revealed, however, that considerable confusion exists in the classification of the leopard frogs or frogs of this type resident in the eastern states and possibly over a wider area. Differences between those forms resident in northeastern and those in the southeastern states have long been recognized, but it appears that sufficient material from a variety of localities has never been examined to make a conclusive analysis of the species. A brief reference to the writings of a few authorities on Salientia classification will serve to illustrate this confusion.

It should be recalled that the southern forms used in these experiments were collected in the vicinity of Philadelphia and the northern forms in northern Vermont. From its place of collection, the southern form doubtless coincides in appearance with that type early described by Schreber (1782) as *Rana pipiens* (Kauffeld 1936 and 1937). Later, Cope (1889), from examination of forms collected in a variety of localities, chose to describe the leopard frogs under three subspecies. The southernmost type he called *Rana virescens sphenocephala;* the type from the Atlantic coast *Rana virescens virescens* (probably same as Schreber's *R. pipiens* and the Philadelphia type of this study); and the type of northern distribution he called *Rana virescens brachycephala* (his description of which coincides perfectly with the northern form used in these experiments). More recent authors (Wright, 1933; Dickerson, 1906) have pictured and described the northern form as the typical *R. pipiens* and both it and the southern form have been considered as such by teachers and investigators alike. Most recently Cope's nomenclature has in part been revived, only instead of using a subspecies classification, the three types have been placed in separate species. Thus the most southern form is called *R. sphenocephala,* the Philadelphia form falls within the range of *R. pipiens* and the northern becomes *R. brachycephala* (Kauffeld, *loc. cit.,* and Stejneger and Barbour, *loc. cit.*).[5] It was on the basis of this latter classification that the frogs were originally considered to represent two species, *R. pipiens* and *R. brachycephala.* It is clear, however, that this classification is uncertain and consideration of some further points increases this uncertainty.

In the first place, it would seem that the two forms hybridize too

[5] In footnote, Stejneger and Barbour (1939) indicate that the whole sphenocephala-pipiens-brachycephala complex needs further examination and possible revision.

successfully to be representatives of two distinct species. It is true that a few distinct species of the Salientia have been successfully hybridized (Born, 1883; Pflüger and Smith, 1883; Heron-Royer, 1891; Montalenti, 1933; Durken, 1938 and Moore, 1940) so that the successful crossing of these two forms, even if they represent distinct species, is not without parallel. What is unique is the result of androgenetic hybridization, for no case involving the Salientia has been reported in which the development of an androgenetic or merogonic species hybrid continued to the advanced stages obtained in these experiments.[6] In other words, the compatibility of the two forms is greater than would be expected of two distinct species.

In the second place, it can be said that the characteristics of the two forms do not differ sufficiently to place them in separate species. Aside from body proportions, which is dealt with below, the major difference is one of pigmentation. This difference, it can be noted, does not involve the pattern but chiefly the size of the markings and intensity of the coloration. These are features which in other animals may vary considerably among races.

Finally, recalling that the two forms were collected from different northerly and southerly climates, and considering the points about to be discussed, the differences in body proportions likewise do not support a species relationship. Taxonomists have long been acquainted with certain generalizations known as the Bergmann and Allen rules pertaining to differences in size and body proportions which can be recognized between the northern and southern races of warm-blooded species. The former of these states that northern races are larger; the latter, that the southern races have relatively longer body projections. Within recent years an increasing volume of research examining racial and subspecies differences has shown that characteristics other than body size and proportions may likewise vary in an orderly and predictable manner with a variety of environmental gradients. Inclusive surveys of these phenomena are to be found in the recent writings of Goldschmidt (1940), Rensch (1936) and others. But among the species of animals examined for chains of racial differences or "clines" (Huxley, 1938), it appears that species of Amphibia have been regrettably absent. Schmidt (1938) reviewed some measurements of species of Salientia and noted that

[6] Baltzer (1920 and 1933) reports that from the combination of *Triton taeniatus* cytoplasm and *Triton palmatus* nucleus heterospermic haploids develop to stages showing good eye formation, pigment, small branching gills, and pulsating heart. Though this represents advanced development as compared with the usual result with different Salientia *species*, the stage reached does not seem to be the equivalent of that reached by the best of the heterospermic haploids obtained with these two *forms* of Rana pipiens.

relative to body size the leg length was greater for those representatives of a species which were collected from the more southern localities. The small number of animals examined and the preserved condition of these did not, however, permit any definite conclusions. Measurements of unselected groups of the two forms used in these crosses show the same tendency of the northern form to have a heavier and larger body structure relative to leg length.

It is possible that a thorough examination of the literature would reveal additional references to racial differences between frogs. For example, such differences are briefly mentioned in a paper by Pflüger and Smith (1883). Comparing the English race of *R. fusca* with the Königsburg race of the same species, they write:

> "Der englische braune Grasfrosch ist etwas kleiner und schlanker als der deutsche, weniger stumpfschnauzig und von zarterer Haut."

The similarity between these differences and those noted between the Vermont and southeastern Pennsylvania forms of *R. pipiens* is obvious. This similarity takes on added interest when it is noted that roughly the same climatic differences (as indicated by mean annual temperatures) exist between East Prussia (44° F.) and England (50° F.) as between Vermont (43° F.) and southeastern Pennsylvania (52° F.).

In view of these observations and the fact that racial variations accompanying climatic gradients have been found in a great many species of both the animal and plant kingdoms, it seems probable that species of frog when thoroughly examined will likewise show various clines with regard to temperature and other environmental factors. In the meantime it can only be maintained that the two forms used in these crosses probably represent two races of the same species.

If such is the case, the results of these experiments are of interest in demonstrating that racial differences involving body proportions can be recognized in early embryonic stages, and that at least some of the factors responsible for these differences exist in the cytoplasmic organization of the egg (see below). This observation and others which will probably be made from a more extensive examination of these and similar crosses may prove of interest to students who concern themselves with factors involved in species formation.

Experiments examining the relative rôles of the nucleus and cytoplasm in heredity have generally shown the nucleus to be the sole bearer of factors controlling the appearance of specific adult and juvenile characteristics. Some of these experiments have combined the nucleus of one species with the cytoplasm of another to form merogonic hybrids, attempting thus to demonstrate the presence of hereditary units in the

cytoplasm. Among these, the studies of the Hertwigs, Boveri, Baltzer, Hadorn, and Hörstadius are well known and frequently reviewed. With the possible exception of Hadorn's (1936) results, the demonstration of cytoplasmic inheritance has not been conclusive. The development of the merogonic hybrids generally ceases very early and even where it continues to a stage showing distinct species characteristics, as in certain sea-urchin merogons, the intermediate condition of the characteristic can be considered as an abnormality resulting from a degree of incompatibility between the nucleus and cytoplasm (Hörstadius, 1936). The early cessation of development which characterizes amphibian merogonic hybrids is probably also the result of a severe incompatibility.

It would appear that by using more closely related forms than those belonging to different species this problem of incompatibility could be overcome. To some extent this is probably true, but in using members of different races or subspecies, it is necessary to sacrifice the clear-cut distinctions which usually exist between the embryonic stages of different species and which are not to be expected between different races. Hence, the problem is fraught with difficulties and it is doubtful whether material such as used in these experiments, though it should be thoroughly examined, will supply any evidence in support of cytoplasmic-borne units of heredity even if present.

As distinct from heredity, cytoplasmic influence on development has been and can be demonstrated. This influence has been considered as the effect of plasmatic organization and composition upon the expression of nuclear factors. To this category of cytoplasmic activity the results of these crosses probably belong. Experimental embryologists have long recognized a high degree of cytoplasmic differentiation in a variety of eggs and the maintenance of such differentiation undisturbed is known to be essential in many cases for normal embryonic development. The cytoplasm of the egg by its organization, therefore, exerts an influence on the appearance of the adult in so far as this appearance is determined by the characteristics of the early developmental stages.[7] Needless to say, the nature of this early cytoplasmic influence is not understood but every new demonstration of its presence offers new possibilities for its examination.

The consideration of the results of these experiments is facilitated if the development of the two control diploids is visualized as paralleling on opposite sides an average or mean type (Fig. 8). If the factors responsible for this slight departure from the mean are nuclear and the

[7] In respect to even this cytoplasmic influence, it is to be remembered that considerable differentiation of the egg takes place in the presence of the maternal nucleus.

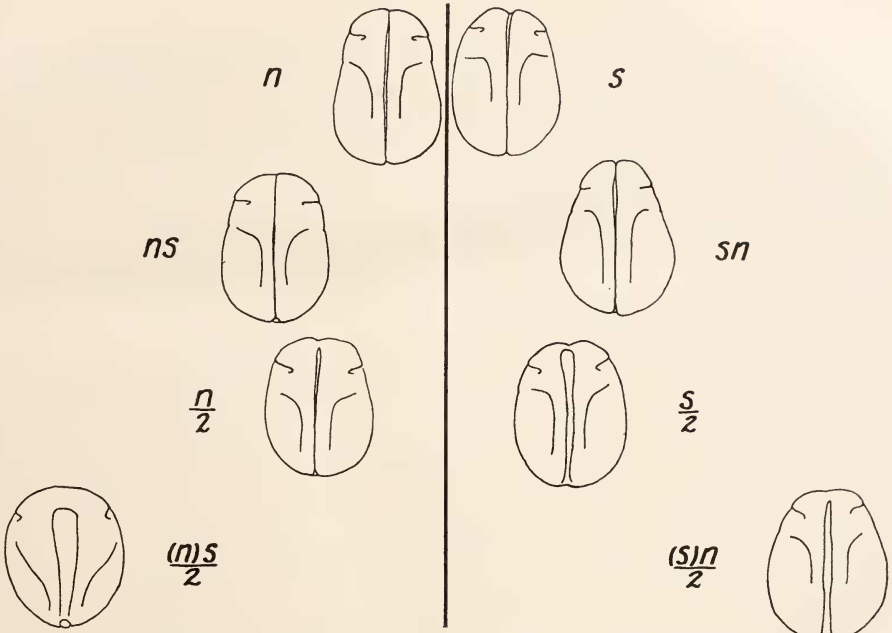

FIG. 8. Dorsal views of 61-hour-old embryos representing the development of the various nuclear-cytoplasmic combinations in relation to a hypothetical mean type, the central line. Those combinations with northern cytoplasm are on the left of the line; those with southern cytoplasm are on the right. The distance from the line represents the approximate degree of difference between the development of any one combination and the mean.

cytoplasms are perfectly neutral to nuclear control, then the diploid reciprocal hybrids would be expected to be identical and would in their development occupy a position coinciding with the hypothetical mean. Under the same conditions of nuclear control, the heterospermic haploid with the southern cytoplasm and northern nucleus would be expected to show the same development as the homospermic haploid of the northern form. Neither of these results is obtained. Instead, it is noted in the case of the reciprocal diploid hybrids that their development places them on opposite sides of the mean and at points more distant from the mean than their diploid controls. And in the case of the heterospermic haploids, the hybrid with the northern nucleus is not only further from the mean than the homospermic haploid of the northern form, but it is on the opposite side. Since the diploid hybrids can be considered as having identical nuclei and differing only in their cytoplasms, and since the same difference holds between the homo- and heterospermic haploids with nuclei of the same form, it follows that cytoplasmic influence is responsible for the dissimilarities existing between them.[8] Therefore, the eggs of the northern and southern forms differ in some property or properties of their cytoplasms.

Are the nuclei identical or do they also differ? If the nuclei are considered as being identical and responding solely to cytoplasmic influence, then the development of the reciprocal diploid hybrids should parallel the mean at the same distance as their respective diploid controls. Or, under the same assumed conditions of identical nuclei, the heterospermic haploid with the southern cytoplasm should be identical in appearance with the homospermic southern haploid. Again, the results indicate that the assumed condition of identical nuclei cannot be valid. On the other hand, the intermediate position of the diploid control between the mean and the hybrid diploid with the same cytoplasm indicates that the nucleus of each race has compensating factors for the cytoplasm of that race. The same conclusion is also supported by the intermediate position of the homospermic haploid relative to the mean and the heterospermic haploid with the same cytoplasm. Evidently then, the nuclei of the two forms also differ and do so in such a way as to compensate in development for cytoplasmic differences.

Cytoplasmic and nuclear differences seemingly demonstrated, it is of interest to determine which is responsible for the slight dissimilarities between the control diploids, and the more distinct dissimilarities between the homospermic (control) haploids of the two races. It has been shown that each diploid control in its morphogenesis is on the same

[8] A heterozygous genome in the parent forms could not account for these differences.

side of the mean as the hybrid diploid with the same cytoplasm, though not at the same distance. The homospermic haploids, in their development, parallel the mean at a greater distance than the control diploids, suggesting a lesser degree of compensation by the haploid nucleus. The homospermic haploid in its morphogenesis shows the same tendencies, though to a lesser degree, as the heterospermic haploid with the same cytoplasm. These facts suggest that the cytoplasmic differences are responsible for the slight dissimilarities between the diploid controls and homospermic haploid controls of the two forms. Further study may demonstrate whether or not these cytoplasmic differences are also related to the dissimilarities of the two adult parent forms.

What is the nature of these nuclear and cytoplasmic differences? There is not, of course, sufficient information available to answer this question. The presence of some degree of cytoplasmic organization in the amphibian egg has been shown to exist as early as 20 minutes after insemination (Fankhauser, 1930) and before first cleavage (Brachet, 1906), but the nature of this organization has not been demonstrated. Though the differences which are being examined cannot be described in precise terms, one feature of their relative nature does become apparent. It is clear from the results that some property or properties of the cytoplasm of the northern form tend to make the embryos with the cytoplasm of that form display certain features of development which, relative to the mean type representing normal development, are the exact opposite of those found in the embryos with the cytoplasm of the southern form (Table II). This infers that the differences in

TABLE II

Northern cytoplasm	Southern cytoplasm
1. *Small* gastrular angle	1. *Large* gastrular angle
2. *Large* completed blastopore	2. *Small* completed blastopore
3. Neural plate abnormally *broad* at anterior end	3. Neural plate abnormally *narrow* at anterior end
4. Neural plate abnormally *short*	4. Neural plate abnormally *long*
5. *Small* tail-bud	5. *Large* tail-bud
6. *Large* head primordia	6. *Small* head primordia
7. *Small* abdomen relative to head size	7. *Large* abdomen relative to head size

organization or composition, whether they be quantitative or qualitative, are of opposite natures as measured in terms of what they tend to produce in development. It has been noted further that the nuclei of the two forms have properties which tend to compensate for the cytoplasmic differences. Therefore the nuclei may also be considered to have properties of opposite nature. If this reasoning is correct, it seems that the nucleus of one form should supplement or enhance the cytoplasmic

influence of the other form. This means that the development of the reciprocal heterospermic haploids should be sufficiently different to suggest the activity of something more than the cytoplasm. While there is no unit of measurement by which the degree of difference can be determined, it is clearly great (Fig. 8) and is probably contributed to by a nuclear influence.

The differences in size of yolk granules and nuclei which preliminary studies have shown to exist between the early embryonic stages of the two forms constitute the only concrete dissimilarities between cytoplasm and nuclei so far observed. What connection, if any, these may have with the actual nuclear and cytoplasmic differences responsible for the above results is not readily apparent.

It is of further interest to determine how these differences operate to produce the results described above. This point is brought into this discussion not because any definite answer can be provided but because certain experimental treatments which could be expected to alter the mode of operation of cytoplasmic and nuclear factors have produced similar results. For example, if a temperature gradient is applied to the developing frog egg, that portion at the warm end of the gradient develops abnormally large structural units (Huxley, 1927; Dean, Shaw, Tazelaar, 1928; Gilchrist, 1928, 1929, 1933). More specifically, if the gradient is applied "adjuvantly" (Huxley, 1927) along the animal-vegetal polar axis in blastula stages (i.e., with warm end of gradient at animal pole) the tail-bud embryos from a blastula so treated have slightly larger heads than the controls and those subjected to the reverse gradient (Huxley and Dean, Shaw, Tazelaar, *loc. cit.*). It is further reported by the same authors that an adjuvant gradient increases by several times the normal difference in size existing between animal and vegetal cells of the blastula stages. Gilchrist (1933) demonstrates that size differences of embryonic structures resulting from temperature gradient treatments are not due solely to age differences but thinks rather that there is an alteration in what he terms the "physiological pattern" of the egg. In this same connection it can be noted that toxic agents applied to developing frog embryos can likewise produce a disproportion of parts most noticeably influencing those regions having the highest metabolic activity at the time of application (Bellamy, 1919).

With these results in mind, it is reasonable to suggest that the differences between the cytoplasms of the eggs of these two geographic forms or races are differences in factors which normally determine the varying rates of metabolism and cell division in the various parts of the developing blastula and possibly the induction processes in later stages. Only one bit of experimental evidence bearing on the physiological

properties of these eggs is available and this of a very preliminary sort—the temperature tolerance is higher for the egg of the southern form. This, it is logical to suppose, is related to the fact that the southern embryos may be called upon to develop at higher temperatures than the northern. From this, however, it is not possible to reason that other physiological differences which may exist between the two eggs are likewise related to climatic influences.

It is realized that other subjects of interest could be discussed in relation to the results of these experiments but it is felt that they may be considered more successfully after more information has been accumulated. For the present, it seems best to emphasize that the gametes of two geographic forms probably of the same species differ slightly in their cytoplasmic and nuclear properties and that by androgenetic haploid as well as diploid hybridization the orderly and measurable effects of these properties on early morphogenesis can be observed. The nature of these differences, their mode of operation, the relation of the embryonic differences they produce to the differences between the adults are among the major problems which can be and should be examined later with the same or similar materials and methods.

Summary

1. Two distinct forms of frog, commonly referred to as *Rana pipiens,* Schreber, are described, and evidence is presented to show that they probably represent geographic races of that species, one from northern Vermont, the other from southeastern Pennsylvania.

2. In the experiments described, the gametes of these two races have been combined reciprocally to form diploid and androgenetic haploid hybrids and the early development of these has been studied in detail.

3. The diploid hybrids developed through metamorphosis; the androgenetic hybrids for 7 to 11 days, up to about stage 24 (Shumway, 1940).

4. A comparison of 3-day-old control and hybrid embryos reveals that, in general, the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such dissimilarities, only slightly apparent between the homospermic diploid controls, become progressively more accentuated between the homospermic haploids, the heterospermic (hybrid) diploids, and the heterospermic (hybrid) haploids.

5. A study of gastrula, neurula, and older stages discloses the early expressions and later fate of the dissimilarities shown by the 3-day-old embryos.

6. These results demonstrate:

(*a*) Cytoplasmic differences between the eggs of the two forms which seem to have contrasting effects upon the same developmental processes.

(*b*) Nuclear differences which, in homospermic diploid control development, appear to compensate for the cytoplasmic differences.

(*c*) An orderly cytoplasmic influence on early morphogenesis.

7. The possible nature and mode of action of these differences are briefly discussed.

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